A STUDY OF VARIOUS METHODS FOR DETERMINING THE VIRULENCE OF DIPHTHERIA BACILLI*

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The virulence of a culture of diphtheria bacilli, as accumulated experience has shown, can be determined only by an animal-inoculation test. Differentiation on morphologic characteristics possesses some value, as experience has shown that the majority of cultures of the granular or beaded and barred types of diphtheria bacilli are virulent, whereas the solid types, particularly the short solid varieties, are nonvirulent; but between these extremes, including cultures falling into each of these divisions, are many types the virulence of which cannot be foretold with any degree of accuracy according to morphology alone. Likewise, sugar-fermentation tests are at best poor guides for determining the virulence of a culture, altho with dextrose and dextrin virulent bacilli invariably produce acids, while the short solid nonvirulent varieties either fail to produce acids with any sugar or do so in a weak and indefinite manner. The only test of value, therefore, is the animal-inoculation test.

These tests are frequently demanded not only in the laboratories of hospitals for contagious diseases, but in board-of-health and other laboratories as well. This is due to the fact that diphtheria-like bacilli may be found in from 5 to 20% of throats and in from 10 to 20% of noses of healthy persons, so that among a number of persons exposed to diphtheria, some will surely show the presence of these micro-organisms. The proper handling of these contacts depends in many instances on whether or not the bacilli are virulent. Generally speaking, from 30 to 80% of cultures of bacilli from the noses and throats of "carriers" who never have had diphtheria, and never have been intimately exposed to a clinical case of this disease, are nonvirulent; on the other hand, bacilli from the noses and throats of persons who have had diphtheria or who have been intimately exposed to the disease are virulent in from 61 to 100% of cases. In the management of these cases, therefore, particularly from the standpoint of quarantine, the history of the

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patient, the type of bacilli found in the cultures, and, finally, a virulence test, are to be considered.

Likewise, afer a patient recovers from an attack of diphtheria, tho in the majority of instances the diphtheria bacilli disappear in from 2 to 5 weeks, bacilli may be present on a mucous membrane over prolonged periods of time. In such case, even when cultures are taken in a most careful manner, 2 consecutive negative cultures may be inferior in value to a virulence test, for additional cultures are frequently positive and hence the patient may be released actually carrying bacilli of unknown virulence.

The demand for a virulence test, therefore, is usually based on good reasons. Justification for such a test and the right to pronounce a culture virulent or nonvirulent, with a full realization of the consequences of permitting a person to mingle freely with others in case of the latter, are based mainly on the following:

1. Accumulated experience tends to show that a properly conducted virulence test may be safely accepted as a criterion of the potential pathogenicity of a culture of diphtheria bacilli. The final test is whether a person harboring bacilli nonvirulent for the test animal is capable of infecting other persons; the experience of many investigators, as well as our own, shows that he is not. But as diphtheria so frequently is spread in a varied and confusing manner absolute statements are not justified.

2. Extensive investigations have generally shown that cultures proving nonvirulent in all tests cannot be made virulent by animal passage or other means. Successful results have been reported but not corroborated. On the other hand, attenuated and slight virulence in a strain may be exalted by animal passage or by transfer of the strain to the mucous membrane of another person, and this has an important bearing on the subject of a virulence test, demanding as it does that the test be made as delicate as possible in order that low degrees of virulence may be revealed.

3. While diphtheria bacilli usually retain virulence both on the mucous membranes and under artificial conditions for long periods of time, yet it is probable that in some instances, at least, this property may be lost. We have long held the opinion, however, that nonvirulent bacilli found on the mucous membrane of healthy "carriers" and convalescents are those which are more or less permanently associated with the individual; that they were present on the mucous membranes before

exposure or an attack of the disease and that they are likely to persist for indefinite periods of time after all clinical evidences of infection have disappeared.

The chief requisites for a reliable virulence test may be summarized as follows:

1. The test must be as delicate as possible in order that it may reveal the potential harmfulness of bacilli of low virulence.

2. Any evidence of virulence, however slight, must be regarded as a positive indication of the pathogenicity of a culture under study, for the conditions governing the virulence test are radically different from those controlling infection among men, and it is better to err on the positive side.

3. The test must be specific, conducted with pure cultures. Cultures contaminated with other micro-organisms may show no evidence of virulence because of alteration of the culture medium or the presence of products of the metabolic activity of the contaminating micro-organisms.

4. The test must be conducted with as large a dose of the culture under study as is possible for specific results and the bacilli must have been cultivated under conditions which are most favorable for rapidity of multiplication and the production of toxin.

5. Because colonies of virulent and nonvirulent bacilli may be present side by side on a solid medium, the test should be conducted with a mixture of the bacilli from 2 or more colonies, or 2 or more colonies should be tested separately.

6. The test must be simple and easily interpreted; also economical and possible of completion in as short a time as is consistent with accuracy.

The guinea-pig is most suitable for virulence tests, not only by reason of its size and general adaptability for laboratory work, but because its tissues are peculiarly sensitive to the influence of the diphtheria bacillus and its toxins.

In the Philadelphia hospital for contagious diseases, where the guinea-pig virulence test is frequently used,[1] this study has been conducted during the past 2 years with the purpose of determining what technic best fulfills the requirements of a satisfactory test.

[1] Kolmer, Woody, and Moshage: Am. Jour. Dis. Child., 1916, 11, p. 257. This is a more complete report of 1,054 of these routine tests.

METHODS OF STUDY

The Routine Virulence Test.—The routine test in our laboratory has consisted, briefly, in cultivating pure growths of bacilli in 0.2% dextrose broth, + 0.8 to phenolphthalein, for 72 hours, and subcutaneously injecting guinea-pigs, weighing from 250 to 300 gm., with a dose of the culture corresponding to 0.5% of the body weight, expressed in cubic centimeters, plus sufficient sterile salt solution to make the total volume 4 c.c.[2]

Cultures for use in this routine virulence test are isolated by the "streak" method on plates of Loeffler's serum media and a number of colonies are studied for purity and for types of bacilli. As shown by Arms and Wade,[3] colonies of virulent and nonvirulent bacilli may be present on the same medium; therefore, in an effort to minimize the chances of conducting our test with a nonvirulent culture, we inoculate broth with 2 or more different colonies. In all comparative tests for virulence, however, the same cultures are used lest one test be conducted with a particular culture and the comparative test with another.

Unless cultures show a good growth in 24 hours, they are transferred one or more times until they have been educated to grow in a fluid medium. Cultures are grown at 35-37 C. with the tubes slanted in order to expose as large a surface as possible to the oxygen within the tube.

After injection, for at least 4 days, the animals are carefully observed for evidences of local edema and general intoxication. Virulent cultures usually kill the test animal within 3 days, while cultures of low virulence produce some edema, which later subsides, loss in weight and appetite, and other symptoms of toxemia.

When in doubt regarding a result, a second guinea-pig is injected with the culture plus diphtheria antitoxin (1 c.c. of a 500-unit serum). Working with known pure cultures of the bacilli, however, and believing that diphtheria-like bacilli may be safely differentiated from other bacilli by morphologic and staining qualities, supported on postmortem examination by the evidences of true diphtheritic intoxication, such as the characteristic local inflammatory reaction, congestion of the suprarenal glands, pulmonary lesions, etc., we do not consider that every culture requires an antitoxin control. We recommend the control inoculation, however, for laboratories in which these tests are conducted only at irregular intervals.

Since the toxicity and the virulence of diphtheria bacilli are not parallel properties and since toxicity alone represents but one pathogenic factor, all our tests are conducted with unfiltered cultures, so that the combined effect of different pathogenic agents may be studied.

As stated, this subcutaneous method constitutes the routine procedure. We have compared other methods with it in an effort to determine the technic which in our opinion is most suitable and reliable.

Classification of Diphtheria Bacilli.—We use the morphologic classification of Wesbrook, Wilson, and McDaniel, but for the sake of brevity all the types of bacilli found in the cultures are not recorded. In a culture of granular bacilli A, B, and C types may be found, but if one predominates, the culture is recorded according to that type. The greater proportion of granular bacilli are of these types, and they seem to be of equal virulence. It is frequently difficult to draw sharp lines among the solid bacilli. Length of incubation and particularly the degree of temperature may modify the appearance of bacilli

[2] Jour. Infect. Dis., 1912, 11, 56. Also Weston and Kolmer: Ibid., 1911, 8, p. 295.
[3] Jour. Am. Med. Assn., 1911, 56, p. 809.

to a considerable extent. The cultures for virulence tests were recorded as follows:

A. Granular bacilli, A, B, C, and D—mostly C.

B. Barred bacilli, A_1, B_1, C_1, and D_1—mostly B_1.

C. Solid bacilli { Long solid, A_2, B_2, and C_2—mostly C_2. / Short solid, D_2 and E_2—mostly D_2.

With the subcutaneous method for determining the virulence of diphtheria bacilli as outlined, were compared the following methods:

1. The intracutaneous injection of 72-hour plain-dextrose-broth cultures.
2. The subcutaneous injection of salt-solution suspensions of 24-hour Loeffler cultures.
3. The subcutaneous injection of 72-hour serum-dextrose-broth cultures as compared with the subcutaneous injection of salt-solution suspensions of 24-hour Loeffler cultures.
4. The subcutaneous injection of 72-hour serum-dextrose-broth cultures.
5. The subcutaneous injection of 9-day plain-dextrose-broth cultures.
6. The intraperitoneal injection of 24-hour plain-dextrose-broth cultures.
7. The intraperitoneal injection of 24-hour serum-dextrose-broth cultures.
8. The intraperitoneal injection of 72-hour plain-dextrose-broth cultures.

RESULTS

The Comparative Virulence of Diphtheria Bacilli in Subcutaneous and Intracutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures.—The results of comparative tests with 37 cultures are shown in Table 1.

In this table the sign + under Subcutaneous Injection indicates that the guinea-pig either died or showed local edema and general toxemia within 4 days after inoculation; the sign + under Intracutaneous Injection indicates the presence of edema and infiltration at the site of injection.

Method.—Pure cultures were grown in plain dextrose broth for 72 hours, and 0.1 c.c. of each culture was injected intracutaneously into a guinea-pig, the skin of the abdomen being used after a spot had been bared by pulling out the hairs. At the same time, a second guinea-pig of proper weight received a subcutaneous injection of the same culture. Both animals were observed for a period of 4 days.

Results as follows were observed: Of the 37 cultures, including granular, barred, and solid types of bacilli, 86.5% were positive for virulence with the subcutaneous method, as compared with 64.9% positive with the intracutaneous method. On several occasions there was difficulty in reading the results of the intracutaneous injections, especially in differentiating between a slight degree of edema and the local

nonspecific inflammatory reaction characterized by a small area of infiltration as met with in Römer's intracutaneous test for toxin.

Neisser has suggested an intracutaneous method for testing the virulence of diphtheria bacilli consisting in the intracutaneous injection of 0.1 c.c. of suspensions of bacilli prepared by emulsifying one loopful

TABLE 1

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRACUTANEOUS INJECTIONS OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	Results	
				Subcutaneous Injection	Intracutaneous Injection
1	Throat	14	Barred (B_2)	+	+
2	Nose	21	Granular (C)	+	—
3	Nose	19	Granular (C)	+	+
4	Throat	30	Long solid (C_2)	+	+
5	Ear	25	Granular (C)	+	+
6	Throat	28	Long solid (C_2)	+	+
7	Ear	25	Granular (C)	+	+
8	Throat	20	Long solid (C_2)	+	+
9	Nose	18	Granular (C)	+	—
10	Nose	22	Long solid (C_2)	+	±
11	Ear	16	Granular (C)	+	—
12	Throat	18	Granular (C)	+	+
13	Throat	16	Long solid (C_2)	+	+
14	Nose	18	Granular (C)	+	+
15	Ear	12	Granular (C)	+	+
16	Throat	18	Granular (B)	+	+
17	Throat	17	Granular (C)	+	+
18	Throat	33	Long solid (C_2)	+	—
19	Ear	41	Granular (C)	+	+
20	Ear	21	Granular (C)	+	—
21	Throat	89	Granular (C)	+	—
22	Nose	18	Long solid (C_2)	—	—
23	Throat	17	Long solid (C_2)	+	+
24	Nose	14	Granular (C)	+	+
25	Nose	8	Granular (C)	+	+
26	Throat	22	Long solid (C_2)	+	—
27	Throat	17	Granular (C)	+	+
28	Ear	22	Granular (C)	+	+
29	Throat	15	Long solid (C_2)	+	+
30	Nose	27	Granular (C)	+	—
31	Nose	15	Short solid (D_2)	—	—
32	Throat	33	Long solid (C_2)	+	+
33	Nose	26	Granular (C)	+	+
34	Throat	25	Granular (C)	+	+
35	Nose	7	Long solid (C_2)	—	—
36	Throat	25	Long solid (C_2)	—	—
37	Throat	18	Long solid (C_2)	—	—

The sign + under subcutaneous injection means that the guinea-pig either died or showed local edema and general toxemia within 4 days after inoculation. Under intracutaneous injection, the sign + means the presence of edema and infiltration at the site of injection. The sign — means that there was no evidence of virulence.

of a 24-hour Loeffler slant in 1 c.c., 10 c.c., and 100 c.c. of normal salt solution. As a control, some antitoxin containing 8 units per cubic centimeter is added to an equal volume of the heaviest suspension, and 0.1 c.c. of this mixture injected intracutaneously into the same guinea-

pig. True virulent bacilli produce an area of superficial necrosis in from 48 to 72 hours, whereas the skin at the site of the control injection should remain normal in appearance. One objection to this method is that in using the same animal for both series of injections the antitoxin may influence the sites of the injection of the culture. More recently Zingher and Soletsky,[4] drawing particular attention to this point, modified Neisser's technic to the extent that 1 guinea-pig receives 3 or 4 injections of different cultures (0.1 c.c. of a 24-hour Loeffler's slant suspended in from 25 to 30 c.c. Nacl solution) while a 2nd guinea-pig, serving as the control, receives injections of the same cultures plus 0.5 c.c. of 200-unit antitoxin, injected intracardially just before or intraperitoneally 24 hours previously. One point strongly in favor of the latter method is that of economy, since 2 pigs suffice for 4 or even 6 tests, the method requiring of course that all injections be made at the same time.

As stated, we have experienced some difficulty in reading the reactions with bacilli of low virulence, so that we prefer a method whereby a sufficient amount of culture is injected to elicit well-marked and definite reactions.

The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures and Subcutaneous Injection of Suspensions of 24-Hour Loeffler Cultures in Sterile Normal Salt Solutions.—The results of comparative tests with 14 cultures are shown in Tables 2 and 8.

Method.—Pure cultures of diphtheria bacilli were grown for 24 hours on slants of Loeffler's blood-serum media in the usual-sized test tubes. After examination of stained smears to insure purity of growth, 10 c.c. of sterile salt solution were used in washing off each culture with the aid of a platinum loop. When a uniform emulsion had been secured, 4 c.c. were injected subcutaneously in the median abdominal line of a guinea-pig weighing from 250 to 300 gm. At the same time the regular subcutaneous test was conducted with a 72-hour plain-dextrose-broth culture of the same micro-organism. All guinea-pigs were observed for a period of 4 days, and if death occurred in any one, it and the control were examined and cultures prepared from the subcutaneous tissues and internal parts.

While the dose was rather large, still in the case of the granular types emulsions of but moderate density had been obtained with 24-hour cultures, and, as previously stated, we believe the test should be conducted in a manner calculated to show low degrees of virulence.

Of 23 cultures, representing granular, barred, long-solid, and short-solid types of diphtheria bacilli, 69.6% were positive for virulence in

[4] Tr. New York Path. Soc., 1915, 15, p. 18. Jour. Infect. Dis., 1915, 17, p. 454.

subcutaneous injection of the emulsion of 24-hour Loeffler cultures, as compared with 65.1% positive in subcutaneous injection of the 72-hour plain-dextrose-broth cultures.

In the case of 10 cultures (Table 2, Nos. 1, 2, 3, 4, 5, 6, and 7; Table 5, Nos. 5, 6, and 9), the results were obtained one or more days

TABLE 2

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Ear	39	Granular (C)
2	Throat	21	Granular (C)
3	Ear	3	Granular (C)
4	Throat	15	Granular (C)
5	Throat	14	Long solid (C_2)
6	Throat	19	Granular (C)
7	Throat	19	Granular (C)
8	Throat	19	Granular (C)
9	Throat	20	Long solid (C_2)
10	Throat	49	Granular (C)
11	Throat	19	Granular (C)
12	Throat	10	Granular (C)
13	Nose	28	Short solid (D_2)
14	Throat	16	Granular (C)

TABLE 3

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR SERUM-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat	15	Granular (C)
2	Throat	3	Long solid (C_2)
3	Ear	18	Granular (C)
4	Throat	16	Granular (C)
5	Ear	28	Granular (C)
6	Ear	30	Long solid (C_2)
7	Nose	28	Short solid (D_2)
8	Throat	19	Granular (C)

sooner with emulsions of 24-hour Loeffler cultures than with the routine method employing 72-hour plain-dextrose-broth cultures. In the case of 4 cultures (Table 2, Nos. 6 and 8; Table 5, Nos. 1 and 2), the results with both methods were obtained on the same day. In one case (Table 2, No. 10) the routine method yielded a positive result one day in advance of that from the 24-hour Loeffler culture.

As shown in Table 5, the subcutaneous injection of this dose of an emulsion of 24-hour Loeffler cultures yielded results equal to those obtained with 9-day cultures of the same bacilli in plain dextrose broth. This method is probably the oldest known, having been originally employed by Loeffler, and, as shown in the preceding and following

TABLE 2—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

24-Hour Salt-Solution Suspension						72-Hour Broth Culture					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
284	4	+	0	0	0	247	1.2	—	—	—	+
262	4	—	+	0	0	268	1.3	—	—	—	+
254	4	+	0	0	0	276	1.4	—	—	—	Toxic
268	4	+	0	0	0	282	1.4	—	+	0	0
294	4	+	0	0	0	304	1.5	—	+	0	0
272	4	—	+	0	0	305	1.5	—	+	0	0
302	4	—	+	0	0	280	1.4	—	—	+	0
278	4	—	+	0	0	266	1.3	—	+	0	0
308	4	—	—	—	—	280	1.4	—	—	—	—
274	4	—	+	0	0	278	1.4	+	0	0	0
280	4	—	—	—	—	276	1.4	—	—	—	—
294	4	—	—	—	—	285	1.4	—	—	—	—
280	4	—	—	—	—	294	1.5	—	—	—	—
288	4	—	+	0	0	298	1.5	—	—	—	Toxic

TABLE 3—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR SERUM-DEXTROSE-BROTH CULTURES AND SALT-SOLUTION SUSPENSIONS OF 24-HOUR LOEFFLER CULTURES

24-Hour Salt-Solution Suspension						72-Hour Serum-Dextrose Broth Culture					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
265	4	—	+	0	0	255	1.3	—	+	0	0
258	4	—	—	—	—	292	1.5	—	—	—	—
270	4	—	+	0	0	268	1.5	—	—	+	0
256	4	+	0	0	0	276	1.4	—	+	0	0
294	4	—	—	+	0	295	1.5	—	—	—	Toxic
300	4	—	—	—	—	280	1.4	—	—	—	—
295	4	—	—	—	—	270	1.4	—	—	—	—
280	4	+	0	0	0	290	1.5	—	—	—	Toxic

tables, it is proved in our experience to be the best, as it saves time spent in performing and reporting on the test and has proved equal or superior to all other methods, in point of delicacy.

Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Serum-Dextrose-Broth Cultures and Salt-Solution Suspensions of 24-Hour Loeffler Cultures.—These comparative tests

TABLE 4

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SERUM-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat	19	Granular (C)
2	Ear	32	Granular (C)
3	Throat	21	Long solid (C_2)
4	Nose	28	Short solid (D_2)
5	Throat	14	Granular (C)
6	Throat	23	Granular (C)
7	Ear	14	Long solid (C_2)
8	Throat	34	Barred (B_1)

TABLE 5

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF SALT-SOLUTION SUSPENSIONS OF LOEFFLER CULTURES; AND OF 72-HOUR AND 9-DAY PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	Salt-Solution Suspension					
				Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
1	Throat	16	Granular (C)	284	4	+	0	0	0
2	Throat	21	Long solid (C_2)	255	4	+	0	0	0
3	Nose	18	Granular (C)	334	4	+	0	0	0
4	Nose	20	Long solid (C_2)	380	4	—	—	—	—
5	Nose	26	Barred (B)	304	4	+	0	0	0
6	Throat	19	Granular (C)	276	4	—	+	0	0
7	Nose	30	Short solid (D_2)	370	4	—	—	—	—
8	Nose	12	Short solid (D_2)	280	4	—	—	—	—
9	Throat	21	Granular (C)	290	4	—	+	0	0

TABLE 6

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat	31	Granular (C)
2	Nose	8	Long solid (C_2)
3	Nose	20	Granular (C)
4	Throat	19	Granular (C)
5	Throat	14	Long solid (C_2)
6	Throat	18	Granular (C)
7	Throat	19	Granular (C)
8	Throat	19	Granular (C)
9	Throat	25	Granular (C)
10	Throat	19	Granular (C)
11	Throat	22	Long solid (C_2)
12	Throat	49	Granular (C)
13	Throat	10	Granular (C)
14	Nose	28	Short solid (D_2)
15	Throat	19	Long solid (C_2)

TABLE 4—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES AND SERUM-DEXTROSE-BROTH CULTURES

Plain Dextrose Broth				Serum Dextrose Broth			
1 Da.	2 Da.	3 Da.	4 Da.	1 Da.	2 Da.	3 Da.	4 Da.
—	+	0	0	—	+	0	0
+	0	0	0	—	+	0	0
—	—	—	—	—	+	0	0
—	—	—	—	—	—	—	—
—	—	—	+	—	+	0	0
—	—	—	Toxic	—	—	+	0
—	—	—	—	—	—	—	—
—	—	—	Toxic	—	—	—	+

TABLE 5—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF SALT-SOLUTION SUSPENSIONS OF LOEFFLER CULTURES; AND OF 72-HOUR AND 9-DAY PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures						9-Day Plain-Dextrose-Broth Cultures					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
275	1.4	+	0	0	0	0	0	0	0	0	0
275	1.4	+	0	0	0	0	0	0	0	0	0
280	1.4	—	—	—	—	300	1.5	—	—	—	Toxic
375	1.4	—	—	—	—	300	1.5	—	—	—	—
280	1.4	—	—	—	+	0	0	0	0	0	0
290	1.5	—	—	—	+	0	0	0	0	0	0
250	1.3	—	—	—	—	250	1.3	—	—	—	—
270	1.4	—	—	—	—	280	1.4	—	—	—	—
285	1.4	—	—	—	Toxic	270	1.4	—	—	—	+

TABLE 6—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)						14-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
257	1.3	—	+	0	0	250	1.3	—	—	—	+
280	1.4	—	—	—	—	260	1.3	—	—	—	—
315	1.6	—	—	+	0	255	1.3	—	—	—	—
282	1.4	—	+	0	0	288	1.4	—	+	0	0
300	1.5	—	—	+	0	318	1.6	—	+	0	0
252	1.3	—	+	0	0	285	1.4	+	0	0	0
305	1.5	—	+	0	0	278	1.4	—	+	0	0
280	1.4	—	—	+	0	304	1.5	—	+	0	0
294	1.5	—	+	0	0	304	1.5	—	—	+	0
266	1.3	—	+	0	0	294	1.5	—	+	0	0
280	1.4	—	—	—	—	288	1.4	—	—	—	—
278	1.4	+	0	0	0	290	1.5	—	—	—	Toxic
294	1.4	—	—	—	—	275	1.4	—	—	—	—
300	1.5	—	—	—	—	248	1.2	—	—	—	—
290	1.5	—	+	0	0	260	1.3	—	—	—	—

were conducted with 8 different cultures in the same manner as the preceding tests, except that serum-dextrose broth was employed instead of plain dextrose broth. The results are shown in Table 3.

The addition of serum to dextrose broth (sterile horse serum 1 part, dextrose broth 3 parts) results in richer growths of all types of diphtheria bacilli. As shown in Table 3, the use of the serum broth with the routine method yielded the same percentage (62.5) of positive reactions, as did the emulsions of 24-hour Loeffler cultures in normal salt solution. The latter method, however, showed a slight superiority in that for 4 (Nos. 3, 4, 5, and 8), or 50%, of the cultures the results with this method were known one or more days before the results with the usual method and were also more definite (Nos. 5 and 8).

The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour Plain-Dextrose-Broth Cultures and Serum-Dextrose-Broth Cultures.—The superiority of serum broth over plain broth as a medium for cultivating diphtheria bacilli for virulence tests is further indicated by comparative tests with 8 cultures, the results being shown in Table 4. In all tests the same cultures were used after cultivation in the same incubators for the same length of time.

As shown in Table 4, the serum-dextrose-broth cultures yielded 6 or 75% positive reactions, as compared with 4 or 50% positive reactions with cultures in plain-dextrose broth. The superiority of serum over plain dextrose broth as a culture medium is also indicated in that earlier (Nos. 5 and 6) or more definite (Nos. 6 and 8) results were obtained with the serum-broth cultures.

Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of Salt-Solution Suspensions of 24-Hour Loeffler Cultures, and of 72-Hour and 9-Day Plain-Dextrose-Broth Cultures.—Since the maximal toxin-production by diphtheria bacilli is not usually secured in less than 5 days, we have compared the virulence of 9 cultures, using the regular 72-hour test and 9-day cultures in plain dextrose broth.

Method.—Cultures were grown in plain dextrose broth under identical conditions for 72 hours and for 9 days, and injected subcutaneously into guinea-pigs weighing from 250 to 300 gm., according to the general rule. At the same time, for comparison, guinea-pigs were injected with 24-hour Loeffler growths of the same cultures.

The results, shown in Table 5, may be summarized as follows: The 9-day cultures yielded 66.6% positive reactions as compared with 45.5% positive reactions with the 72-hour cultures. This superiority of 9-day cultures was to be expected, but no superiority over the sub-

cutaneous injection of 24-hour Loeffler cultures was shown. Furthermore, the longer time required to conduct a virulence test with 9-day cultures counts against the value of the method for routine work, altho this drawback would be of less significance if greater delicacy were obtained.

The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour, and Intraperitoneal Injection of 24-Hour, Plain-Dextrose-Broth Cultures.—Since absorption from the peritoneal cavity is more rapid than from the subcutaneous tissues, we have sought to determine whether this route of inoculation would render a virulence test more delicate while at the same time shortening the time required for a result.

Method.—Pure cultures of the same bacilli were grown in tubes of plain dextrose broth for 24 and 72 hours under identical conditions, and guinea-pigs were injected intraperitoneally and subcutaneously with doses proportioned to body-weight, in accordance with our general rule.

The results, shown in Tables 6 and 9, may be summarized as follows: Of 27 cultures tested for virulence by these methods, 74% yielded positive results in subcutaneous injection of 72-hour plain-dextrose-broth growths, as compared with 63% positive reactions in intraperitoneal injection of corresponding doses of 24-hour growths in the same medium. In the case of 7 cultures (Table 6, Nos. 4, 7, and 10; Table 9, Nos. 1, 4, 5, and 9) the positive results with both methods were observed at the same intervals after injection; for 5 cultures (Table 6, Nos. 1 and 9; Table 9, Nos. 3, 8, and 9) the subcutaneous method yielded an earlier result; for only 2 cultures (Table 6, Nos. 5 and 8) did the intraperitoneal method yield a quicker result. These results show definitely the superiority of the routine subcutaneous test with 72-hour cultures over the intraperitoneal injection of 24-hour cultures.

The Comparative Virulence of Diphtheria Bacilli in Subcutaneous Injection of 72-Hour, and Intraperitoneal Injection of 24-Hour, Serum-Dextrose-Broth Cultures.—As the addition of serum to broth enhances the growth of diphtheria bacilli, we have tested the comparative delicacy of intraperitoneal injections with serum-broth cultures, the technic being the same as that described for the plain-broth cultures. The results are shown in Table 7.

In the case of 8 cultures tested, including granular, long solid, and short solid types of bacilli, the results were about equal with both methods in that both showed virulent bacilli for 50% of the cultures.

TABLE 7

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, SERUM-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Throat	15	Granular (C)
2	Throat	3	Long solid (C_2)
3	Ear	18	Granular (C)
4	Throat	16	Granular (C)
5	Ear	28	Granular (C)
6	Nose	21	Short solid (D_2)
7	Throat	19	Granular (C)
8	Throat	17	Granular (C)

TABLE 8

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus
1	Nose	8	Short solid (D_2)
2	Ear	19	Short solid (D_2)
3	Ear	32	Short solid (D_2)
4	Nose	20	Granular (C)
5	Throat	31	Granular (C)
6	Nose	30	Granular (C)
7	Ear	39	Granular (C)
8	Ear	21	Granular (C)
9	Throat	19	Granular (C)
10	Throat	25	Granular (C)
11	Throat	19	Granular (C)
12	Throat	19	Granular (C)
13	Nose	28	Short solid (D_2)
14	Throat	18	Long solid (C_2)
15	Throat	42	Short solid (D_2)

TABLE 9

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

Number	Source	Day of Disease	Type of Bacillus	72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)					
				Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
1	Ear	25	Granular (C)	260	1.3	+	0	0	0
2	Nose	125	Granular (C)	256	1.3	—	+	0	0
3	Nose	40	Granular (C)	243	1.2	—	+	0	0
4	Ear	27	Granular (C)	256	1.3	+	0	0	0
5	Ear	31	Granular (C)	261	1.3	+	0	0	0
6	Nose	30	Long solid (C_2)	241	1.2	—	+	0	0
7	Throat	14	Long solid (C_2)	300	1.5	—	+	0	0
8	Nose	17	Granular (C)	273	1.3	+	0	0	0
9	Throat	21	Granular (C)	248	1.3	—	+	0	0
10	Nose	18	Long solid (C_2)	260	1.3	—	—	—	—
11	Throat	4	Long solid (C_2)	263	1.3	—	—	—	—
12	Throat	23	Granular (C)	269	1.3	—	—	—	—

TABLE 7—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, SERUM-DEXTROSE-BROTH CULTURES

72-Hour Serum-Dextrose-Broth Cultures (Subcutaneously)						24-Hour Serum-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
255	1.3	—	+	0	0	252	1.3	—	+	0	0
292	1.5	—	—	—	—	280	1.4	—	—	—	—
268	1.3	—	—	+	0	275	1.4	—	+	0	0
276	1.4	—	+	0	0	294	1.5	—	+	0	0
295	1.5	—	—	—	Toxic	249	1.3	—	—	—	+
300	1.5	—	—	—	—	270	1.4	—	—	—	—
280	1.4	—	—	+	0	285	1.4	—	+	0	0
276	1.4	—	—	—	+	300	1.5	+	—	—	—

TABLE 8—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF 72-HOUR PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Subcutaneously)						72-Hour Serum-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.
280	1.4	—	—	—	—	274	1.4	—	—	—	—
260	1.3	—	—	—	—	263	1.3	—	—	—	—
305	1.5	—	—	—	—	295	1.5	—	—	—	—
240	1.2	—	+	0	0	250	1.25	—	+	0	0
257	1.3	—	+	0	0	256	1.3	—	—	+	0
315	1.6	—	—	+	0	264	1.3	—	—	+	0
284	1.4	—	—	—	+	262	1.3	—	—	—	—
268	1.3	—	+	0	0	276	1.4	—	—	—	Toxic
266	1.3	—	+	0	0	286	1.4	—	+	0	0
294	1.5	—	+	0	0	268	1.3	—	+	0	0
254	1.3	—	—	+	0	270	1.4	—	+	0	0
276	1.4	—	—	—	—	272	1.3	—	—	—	—
294	1.5	—	—	—	—	254	1.3	—	—	—	—
300	1.5	—	—	—	Toxic	318	1.6	—	—	—	—
284	1.4	—	—	—	—	290	1.5	—	—	—	—

TABLE 9—*Continued*

COMPARATIVE VIRULENCE OF DIPHTHERIA BACILLI IN SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OF 72-HOUR, AND INTRAPERITONEAL INJECTION OF 24-HOUR, PLAIN-DEXTROSE-BROTH CULTURES

72-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)						24-Hour Plain-Dextrose-Broth Cultures (Intraperitoneally)					
Weight in Gm.	Dose, c.c.	1 Da.	2 Da.	3 Da.	4 Da.	Weight in Gm.	Dose, s.s.	1 Da.	2 Da.	3 Da.	4 Da.
300	1.5	+	0	0	0	287	1.4	+	0	0	0
300	1.5	—	—	—	—	300	1.5	—	—	—	—
280	1.4	—	—	+	0	250	1.3	—	—	—	+
300	1.5	+	0	0	0	240	1.2	+	0	0	0
295	1.5	+	0	0	0	240	1.2	+	0	0	0
246	1.2	+	0	0	0	250	1.3	—	+	0	0
282	1.4	—	—	+	0	240	1.2	—	+	0	0
272	1.4	—	—	+	0	260	1.3	—	—	—	+
261	1.3	+	0	0	0	240	1.2	—	+	0	0
249	1.3	—	—	—	—	280	1.4	—	—	—	—
244	1.2	—	—	—	—	260	1.3	—	—	—	—
246	1.3	—	—	—	—	280	1.4	—	—	—	—

In the case of 4 cultures (Nos. 3, 5, 7, and 8) the intraperitoneal injection of 24-hour serum-broth cultures yielded quicker or more positive results than did the subcutaneous injection of 72-hour plain-broth cultures. As shown by these tests, therefore, the intraperitoneal injection of 24-hour serum-broth cultures as a method, is equal to the subcutaneous injection of 72-hour cultures and somewhat superior, in that less time is required for the conduct of the virulence test.

Comparative Virulence of Diphtheria Bacilli in Subcutaneous and Intraperitoneal Injection of 72-Hour Plain-Dextrose-Broth Cultures.—Further to study the possible superiority of the intraperitoneal over the subcutaneous route for conducting virulence tests, we tested a number of cultures by both methods, the technic being similar to that already described. The results are given in Tables 8 and 9.

TABLE 10

A SUMMARY OF RESULTS REGARDING THE VIRULENCE OF DIPHTHERIA BACILLI AS DETERMINED BY VARIOUS METHODS

Total Examined	Culture Medium	Hours of Incubation	Route of Injection	Percentage of Positive Results
37	Plain Dextrose Broth.....	72	Subcutaneous.....	86.5
22	Plain Dextrose Broth.....	72	Subcutaneous.....	63.6
8	Serum Dextrose Broth.....	72	Subcutaneous.....	62.5
27	Plain Dextrose Broth.....	72	Subcutaneous.....	74.0
8	Serum Dextrose Broth.....	72	Subcutaneous.....	75.0
27	Plain Dextrose Broth.....	72	Subcutaneous.....	63.0
9	Plain Dextrose Broth.....	72	Subcutaneous.....	45.5
8	Plain Dextrose Broth.....	72	Subcutaneous.....	50.0

In the case of 27 cultures tested, including granular, long solid and short solid types, the subcutaneous injection of 72-hour growths in plain dextrose broth yielded 63% positive results as compared with 55.5% positive results with the intraperitoneal injection of 72-hour plain-dextrose-broth cultures of the same bacilli cultivated under the same conditions. Usually the positive results were observed just as early with the subcutaneous, as with the intraperitoneal, injections. These results indicate that the intraperitoneal injection of plain-dextrose-broth cultures offers no advantages over the subcutaneous route.

SUMMARY AND CONCLUSIONS

A summary of these comparative virulence tests is shown in Table 10. The results of the study and the conclusions drawn may be stated as follows:

The intracutaneous injection of 0.1 c.c. of 72-hour plain-dextrose-broth cultures of diphtheria bacilli proved inferior to the subcutaneous injection of the same cultures into guinea-pigs, weighing from 250 to 300 gm., in dose corresponding to 0.5% of the body weight expressed in cubic centimeters. The former method yielded 64.9% positive results, as compared with 86.5% with the latter method.

It is more difficult to read and interpret the results with the intracutaneous method than with the subcutaneous method.

Of all the methods employed, that of subcutaneous injection of suspensions of 24-hour Loeffler cultures in normal salt solution yielded the best results. This method yielded 69.6% positive results as compared with 65.1% positive results, with the subcutaneous injection of 72-hour plain-dextrose-broth cultures.

TABLE 10—*Continued*

A SUMMARY OF RESULTS REGARDING THE VIRULENCE OF DIPHTHERIA BACILLI AS DETERMINED BY VARIOUS METHODS

Culture Medium	Hours of Incubation	Route of Injection	Percentage of Positive Results
Plain Dextrose Broth	72	Intracutaneous	64.9
Loeffler	24	Subcutaneous	68.1
Loeffler	24	Subcutaneous	62.5
Plain Dextrose Broth	24	Intraperitoneal	63.0
Serum Dextrose Broth	24	Intraperitoneal	75.0
Plain Dextrose Broth	72	Intraperitoneal	55.5
Plain Dextrose Broth	9 days	Subcutaneous	66.6
Serum Dextrose Broth	72	Subcutaneous	75.0

The subcutaneous injection of 72-hour serum-broth cultures yielded the same percentage (62.5) of positive reactions as the subcutaneous injection of 24-hour Loeffler cultures, but the latter is a superior method, as it consumes less time, while being just as delicate and positive in its results.

Twenty-four-hour Loeffler cultures in subcutaneous injection yielded the same results as 9-day plain-dextrose-broth cultures.

Serum-dextrose-broth cultures yielded a higher percentage of positive results (75%) than did plain-dextrose-broth cultures (50%) when both were cultivated for the same length of time and injected subcutaneously in equal dosage.

The subcutaneous injection of 72-hour plain-dextrose-broth cultures proved superior to the intraperitoneal injection of 24-hour and 72-hour plain-dextrose-broth cultures. With serum-dextrose-broth cultures, however, the intraperitoneal injection of 24-hour growths was

equal to, if not slightly superior to, the subcutaneous injection of 72-hour cultures.

From the standpoints of delicacy and of time required, the subcutaneous injection of 24-hour Loeffler cultures after the method described, yielded the best results.

The subcutaneous injection of 72-hour serum-dextrose-broth cultures yielded equally good results, but this method requires more time for the conduct of a virulence test; likewise, the intraperitoneal injection of 24-hour serum-dextrose-broth cultures yielded good results, but this method is less to be preferred than subcutaneous inoculation, because with the latter, local inflammatory changes are more easily detected.